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Expression of genes in the PI3K/Akt pathway in canine cortisol-secreting adrenocortical tumors

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ABSTRACT

Conventional surgical and medical treatment of canine cortisol-secreting adrenocortical tumors is associated with a high risk of complications and negative side effects. The need for novel, more effective treatment options is increasing. The development of 'Targeted drugs' might provide new treatment options. The PI3K/Akt/mTOR signaling pathway have been studied in the last years in order to find more effective cancer therapies. In human tumors, dysregulation of multiple components of this pathway has been observed frequently in adrenocortical tumors. Therefore, it is an interesting pathway in canine cortisol-secreting adrenocortical tumors. In this study, the mRNA expression of ErbB1, ErbB2, ErbB3,ErbB4, ID1, ID2, Snail, Slug, Cyclin D1, PTEN, BCL2 and COX 2 was investigated in 20 canine adrenocortical carcinomas, 10 adenomas, and 10 healthy adrenal glands. All tumors were cortisol secreting. These 12 target genes and also 14 reference genes were quantified by means of a quantitative RT-polymerase chain reaction (qPCR). Mann Whitney U tests revealed that there was a significant up-regulation of ErbB2 and Snail in the group of carcinomas, compared to the normal adrenal tissue. The carcinomas showed a significant down-regulation of ErbB3 and ErbB4 in comparison with normal adrenals. The adenomas showed a significant down-regulation of ErbB4 and Cyclin D1. ErbB2 was significantly up-regulated in the carcinomas compared to the adenomas. In samples with a fold change of 0,1 or lower for ErbB2, mutation analysis was performed on exon 15 of bRAF, as mutations in this gene are known to result in a down-regulation of ErbB2. There were no amino-acid chaning mutations found in exon 15 of bRAF in this study, so this cannot be an explanation of the low ErbB3 expression in the sequenced samples. The expression of the genes did not represent an obvious activation of the PI3K/Akt/mTOR pathway.

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1. INTRODUCTION

Canine cortisol-secreting adrenocortical tumors

The adrenal gland consists of a medulla, cortex and capsule. The cortex includes three different zones, the outermost layer zona glomerulosa, the middle -fasciculata and the innermost -reticularis. The zona glomerulosa produces mineralcorticoids like aldosterone, the zona fasciculata glucocorticoids like cortisol and the zona reticularis androgens. An adrenocortical tumor (AT) associated with Cushing's syndrome, or hypercortisolism, is identified by the fact that the tumor causes elevated cortisol levels and consists most of the time of zona fasciculata tissue. Cushing's syndrome is common in dogs (1 to 2 cases/1000 dogs/year) and much less frequently diagnosed in cats and humans. About 15-20% of cases of Cushing's syndrome in dogs are due to benign or malignant ATs.[1, 2,3,4]

Histologically, canine ATs can be classified as adenomas or carcinomas. Unilateral solitary lesions are the most common ATs. In about 10% of the cases, the tumor occurs bilaterally.[1,5,6]Morphological criteria which are significantly associated with canine adrenocortical carcinomas include a size larger than 2cm in diameter, peripheral fibrosis, capsular invasion, trabecular growth pattern, hemorrhage, necrosis and single-cell necrosis. Hematopoiesis, fibrin thrombi and cytoplasmic vacuolation are significantly associated with canine adrenocortical adenomas [6]. Microscopic examination reveals benign canine ATs can expand into blood vessels, which makes the distinction with a malignant tumor difficult to make. [1,5,6]

Some adrenocortical neoplasms and nodules have been detected in patients without symptoms of hormonal abnormalities. That means not every AT leads to clinical symptoms. These tumors are called 'non-functioning ATs'. In veterinary practice, most dogs diagnosed with ATs have a functional AT which causes elevated hormone levels, because dog owners notice the clinical symptoms of active tumors. Some of the symptoms in dogs are not present in other species, but the clinical presentation of dogs and humans with hypercortisolism have many similar features. A lot of symptoms are a result of gluconeogenesis and lipogenesis at the expense of proteins . Cushing's syndrome is characterized by abdominal obesity, gain of weight, fatigue, muscle atrophy and skin changes both in dogs and humans.[1,2,3]

Treatment

For canine ATs, surgical removal (adrenalectomy) represents the treatment of choice. The cause of hypercortisolism is removed with an adrenalectomy. When the adrenal gland is removed on one side there is no need for lifelong medication. However, temporarily glucocorticoid substitution is needed because of the atrophy of the cortex of the remaining adrenal gland. Bilateral adrenalectomy requires lifelong replacement therapy.[1] Adrenalectomy is associated with a peri-operative mortality of about 20%.[7, 8] This is mainly due to a relatively high risk of postoperative complications. It is also a consequence of the difficulty of the surgical procedure . Due to obesity and hepatomegaly in dogs with hypercortisolism, the adrenal glands are poorly accessible. Furthermore, the tumors are friable, lie in close proximity to the vena cava and tend to invade blood vessels, which complicates the surgery.

Inoperable and/or metastasized functional ATs can be medically treated with mitotane (o'p'DDD). The aim of treatment of ATs with mitotane is complete destruction of adrenocortical cells, including those in metastases.[1] Adverse effects as a result of a drug toxicosis associated with administration of a high dose of mitotane, low serum cortisol concentration, or both can occur in dogs which are treated with mitotane.[9] If there is a metastasis of a functional AT or if neither adrenalectomy or adrenocortical destruction with mitotane is an option, trilostane can be used as a palliative treatment. Trilostane is a competitive inhibitor of the enzyme system HSD3B, which is essential for the synthesis of a few hormones, including cortisol.[1] Because conventional medical treatment may have negative side effects and does not interact with cancer cells specifically, the need for novel, more effective treatment options is increasing. The development of 'Targeted drugs' might provide new treatment options. Targeted drugs target cancer specific factors and inhibit or impair their function. These drug leave normal cells and tissues mostly unaffected.

PI3K/Akt/mTOR pathway

The phosphoinositide 3 kinase (PI3K)/ protein kinase B (PKB or Akt)/ mammalian target of rapamycin (mTOR) signaling pathway have been studied in the last years in order to find a more effective cancer therapy. In human tumors, dysregulation of multiple components of this pathway has been observed frequently.[10] Some targeted drugs which target this pathway are already used in human cancer therapies.[11] Therefore, this pathway is an interesting target in cancer treatment. In normal conditions, the binding of growth factors and cytokines to their corresponding receptors is responsible for activating the PI3K/Akt/mTOR pathway. Receptors that use this pathway for signal transduction are receptors for interleukin 1,2,3,4 and 6 (IL-1,2,3,4,6), insulin-like growth factor (IGF), insulin growth factor (IGF-I and II), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor β (TGF-β), and c-KIT. The PI3K/Akt/mTOR signaling pathway regulates several processes essential for cell growth, -proliferation, -survival and angiogenesis. [12]

The ErbB family of receptor tyrosine kinases (RTK's) are receptors which use this pathway for signal transduction. [13,14] The ErbB family consists of four receptors: epidermal growth factor receptor (EGFR/ErbB1/HER1), ErbB2 (neu/HER2), ErbB3 (HER3) and ErbB4 (HER4). In general, ErbB receptors have an extracellular ligand-binding domain, an α-helical transmembrane segment, a cytoplasmic tyrosine-kinase domain and a C-terminal phosphorylation tail.[15] The binding of ligands, such as epidermal growth factors, to their corresponding receptor or a high receptor density owing to overexpression stimulate dimerization. Ligands binding ErbB receptors can be subdivided in neuregulins and EGF-like ligands. The activation of the ErbB receptors is achieved by dimerization of two identical ErbB receptors (homodimerization), or between two different receptors (heterodimerization) of the ErbB family.[13] As a result of dimerization between two ErbB receptors, the intrinsic protein tyrosine kinase is activated and tyrosine is phosphorylated.[13,14] ErbB3 can only form heterodimers and has no intrinsic kinase activity. This explains why mutual interactions of the receptors are crucial. As far as known, no ligand binds to the ErbB2 receptor with high affinity, however this receptor functions as an important and potent co-receptor for ErbB3 and ErbB4 and is also a preferred co-receptor for ErbB1. In these heterodimers, ErbB2 decelerates the rate of ligand dissociation.[13,15,16] The variety of ErbB combinations in a dimer receptor complex can trigger a complex network of signalling pathways.[13,14] The RAS/RAF/MAPK pathway and the PI3K/Akt pathway

are two important pathways which are activated by the ErbB receptors.[15] The RAS/RAF/MAPK pathway induces cellular proliferation and mediates the growth-stimulating effect of activated receptor tyrosine kinases.[17]

When the PI3K/Akt/mTOR pathway is activated, the enzyme PI3K is recruited to the cell membrane and catalyzes phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3). This conversion creates docking sites on PIP3 for signaling proteins like 3-phosphoinositide-dependent protein kinase 1(PDK1) and Akt. Phosphatase and tensin homologue (PTEN) limits the signaling by dephosphorylating PIP3 back to PIP2. Akt is activated through phosphorylation by serine-threonine kinase PDK1 and TORC2. P-Akt activates many downstream signaling cascades. Activation of Akt eventually leads to activation of the mTOR1 complex and inhibitory phosphorylation of forkhead family of transcription factors, subclass O (FoxO).[18,19]

FoxO can activate a number of cell cycle regulatory proteins, but can also promote cell cycle arrest through the inhibition of D-type cyclins. The expression of inhibitor of DNA binding protein 1 (ID1) is suppressed by direct binding of FoxO3a (one of the four isoforms of FoxO) to its promoter region [20]. Inhibitor of DNA binding protein 2 (ID2) is stimulated by FOXO1.[21] ID proteins have the ability to stimulate proliferation and to inhibit differentiation of certain lineages.[22] Family members of Bcl-2 are stimulated by Foxo. The Bcl-2 family plays a critical role in regulation cell survival and has pro- an anti-apoptotic members.[23]

The expression of the transcription factors Slug and Snail, members of the zinc-finger transcription factor family, can also be regulated by the PI3K/Akt/mTOR pathway. mTOR induces the transcription of Snail and Slug, which are both Snail family genes and repress E-Cadherin (endothelial cadherin) . Snail is also a downstream target of the Wnt-pathway.[24,25,26,27] The function of E-cadherin is to maintain cell-cell contact. Repression of E-cadherin by Snail and Slug leads to epithelial to mesenchymal transition.[26] Epithelial to mesenchymal transition is thought to play an important role in tumor progression and metastatic spread. [27]

The expression of cyclooxygenase 2 (COX-2) can be up-regulated via the MAKP pathway.[28, 29] In addition, COX-2 can influence the PI3K/Akt/mTOR pathway. Most solid tumors exhibit pro-inflammatory properties and exhibit levels of prostaglandins (PG's) due to some pro-inflammatory physiological conditions in tumors, such as hypoxia and a low extracellular pH value. COX-2 is an important enzyme in the cyclooxygenase pathway which is expressed in the cytoplasm of many cell types in response to pro-inflammatory stimuli. The cyclooxygenase pathway leads to the synthesis of prostaglandins, proteoglycans and thromboxanes, which have effects on several important pathways, including the PI3K/Akt/mTOR pathway[30,31] Proteoglycans together with prostaglandins can promote phosphorylation of Akt.[32]

The aim of this research was to investigate whether components of the PI3K/Akt/mTOR signalling pathway were up/down-regulated in canine cortisol-secreting adrenocortical carcinomas and adenomas in comparison with normal adrenals. The target genes of interest were ErbB1, ErbB2, ErbB3,ErbB4, ID1, ID2, Snail, Slug, Cyclin D1, PTEN, BCL2 and COX 2. It was expected that the expression of the target genes would indicate an activation of the PI3K/Akt/mTOR pathway .

2.MATERIALS AND METHODS

2.1 Canine tissues

Tissue samples from 20 canine adrenocortical carcinomas, 10 adenomas, and 10 healthy adrenal glands were used in this research. The normal adrenal glands which serve as control tissues were derived from healthy beagle dogs. The 30 ATs were obtained from dogs with ACTH-independent hypercortisolism. They all underwent unilateral adrenalectomy at the Department of Clinical Sciences of Companion animals in Utrecht. Their ages ranged from 6 to 14 years at the time of surgery. The dogs were suspected of having hypercortisolism based on the history, physical examination and routine laboratory findings. The diagnosis of hypercortisolism was considered to be confirmed by finding of an urinary corticoid:creatinine ratio (UCCR) above the upper limit of the reference range (>8.3 x 10^{-6}) in two consecutive morning urine samples collected at home. The dogs received three oral doses of 0.1 mg dexamethasone per kg body weight at 8 h intervals after collection of the second urine samples. The following morning the third urine sample was collected. The diagnosis ACTH-independent hypercortisolism was confirmed when the third UCCR sample was suppressed by less than 50% of the mean of the first two samples and when the basal plasma ACTH concentration was suppressed (lower than 15 ng/L). The ATs were visualized by abdominal ultrasonography or computed tomography in all dogs. Histopathological examination confirmed the diagnosis of AT. The criteria for ATs to be considered a carcinoma were: a tumor size exceeding 2 cm in diameter, histological evidence of invasion of neoplastic cells into blood vessels, peripheral fibrosis, capsular invasion, a trabecular growth pattern, hemorrhage, necrosis and single cell necrosis. Criteria for adenomas were: hematopoiesis, fibrin thrombi and cytoplasmic vacuolization.[33]

2.2 RNA isolation

The 40 adrenal tissue samples were stored at -80^{0}C until use for RNA isolation. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate the RNA from the ATs and normal adrenal glands. The protocol 'Purification of total RNA from animal tissues' was followed (appendix 1). The frozen tissues were disrupted and homogenized in Buffer RLT using a rotor-stator homogenizer. After centrifuging the lysate, the supernatant was removed and 70% ethanol was added to the cleared lysate and mixed by pipetting. Then, the sample was transferred to an RNeasy spin column, placed in a 2ml collection tube and centrifuged. The optional on-column DNase digestion protocol with the RNase-free DNase set was followed to remove residual DNA (appendix 1). After this extra step, RPE buffer was added to the RNeasy spin column and centrifuged. After discarding the flow through, RPE buffer was added to the spin column and centrifuged again. Then, the spin column was placed in a new 2 ml collection tube and centrifuged. The spin column was placed in a new 1,5 ml collection tube, RNase-free water was added on the spin column membrane and was centrifuged. The concentrations of isolated RNA samples were measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260-A280 ratio of the measured RNA had to be 1,8-2,0. With a ratio of 1,8-2,0, there is a good balance between RNA and proteins.

2.3 cDNA synthesis

The cDNA was made using the iScript^{TM}cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Appendix 2 gives an overview of the protocol and reaction setup. Appendix 3 gives an overview of the all the samples that were used. For cDNA synthesis, a starting quantity of 1000ng RNA was used. This means that the amount of isolated RNA solution which has to be added in the tube differs in each sample. When the RNA concentration in a sample was very very high, it was necessary to dilute the RNA sample 10x. mQ was added to 1000ng RNA to add up to a volume of 15ul in each tube. The RNA concentrations and the quantities of RNA and milliQ (mQ) which were used can be found in appendix 3. Thereafter, iScript Reaction Mix and iScript reverse transcriptase were added to each sample.

The tubes with reverse transcriptase were the reverse transcriptase positive (RT +) tubes. Reverse transcriptase negative (RT-) tubes of each sample were also made to monitor for potential DNA contamination. These tubes did not contain reverse transcriptase. The reaction setup of appendix 2 was used to make the RT-, but milliQ was added to the tube instead of reverse transcriptase.

Finally, the RT+ and RT- tubes of the 40 samples were placed in the PCR C1000^{TM} Thermal Cycler (Bio-Rad, CA, USA), set according to the protocol provided with the iScript kit.

2.4 qPCR

14 reference genes and 12 target genes were quantified by means of a quantitative RT-polymerase chain reaction (qPCR). The target genes of interest were ErbB1, ErbB2, ErbB3,ErbB4, ID1, ID2, Snail, Slug, Cyclin D1, PTEN, BCL2 and COX 2. The reference genes were GAPDH, HPRT, RPS19, HNRPH, RPL8, GUSB, RPS5, SRPR, B2MG, HMBS, RPS13, SDHA, YWHAZ and TBP. A stable set of reference genes was selected based on the results in geNorm and REST 2008. The stable set of reference genes had no significant differences between the carcinoma-, adenoma- and normal sample group and were stable within each sample group. The primers of the reference- and target genes were all present at the faculty of veterinary medicine in Utrecht. The primers were already used in earlier experiments and were validated by means of qPCR temperature gradients and sequencing. Appendix 4 gives an overview of the primer sequences and optimal annealing temperatures.

The Protocol 'qPCR iQSYBRgreensupermix' was followed to prepare the qPCR reactions (appendix 5). The cDNA of all samples had to be diluted before it was used for a qPCR. First, the sample dilution was made by diluting the cDNA 10x. 3 µl was extracted of each sample to form the standard pool. The highest standard (S1) consisted of all of the 97 samples (40 adrenal tissue samples and 57 insulinoma samples of another research). The standard samples ranged from Standard 1 (S1) to Standard 8 (S8). The serial dilution for the standard line was a 4 fold dilution. There were also 4 RT- samples made, which consisted of a pool of 10 random selected adrenal tissue RT- samples. Finally, a 50x dilution of cDNA was needed. The cDNA was already 10x diluted, so a 5x dilution was made of the 10x dilution. One sample functioned as no template control and contained mQ. It functioned as a control sample for cDNA contamination.

After making the dilutions, the master mix was made, which contained mQ, SYBR green Supermix and de forward- and reverse primer. (Appendix 5 gives an overview of the master mix). Finally, the master mix and cDNA was pipetted into a 384 wells plate (See appendix 5 for an overview of the

wells plate) with a reaction volume of 10 µl per well. All reactions were run on a CFX 384™Real-Time PCR System (Bio-Rad, CA, USA).

2.5 Calculations

Bio-Rad CFX Manager™ 3.0 software was used to process results. The Ct values of a sample give information about the expression of the gene of interest in that sample. The higher a Ct value, the more cycles were needed to detect the gene of interest in that sample. So samples with a high Ct value have a low relative gene expression.

The reverse transcriptase negative (RT-) samples of each gene were checked. If the Ct values of the RT- samples differ less than 10 Ct values compared to the RT+ samples, then the Ct values of the samples of a gene are not reliable. A difference between the RT- and RT+ samples of less than 10 Ct values indicates genomic DNA contamination of the isolated RNA. Also the no template controls (NTC's) were checked . A Ct value of the NTC indicates contamination of the mQ with cDNA. The efficiency also gives an important indication of the reliability of the results. The efficiency may vary from 90% to 120%. Melting curve analysis is essential for the identification of non specific products. The melting curve of the qPCR should have only one peak in a specific qPCR assay.

The changes in relative gene expression were analyzed for all target genes. First, the geometric means of all selected reference genes were calculated for each sample. The geometric means of the selected reference genes were used to normalize the results of the target genes. The ΔΔ Ct method was used for further calculations.[34]

2.6 Statistical analysis

The expression levels of the target genes in carcinomas, adenomas and normal adrenal tissues were compared. Because the data is not normally distributed, a non-parametric test was used. The three groups of tissues were compared in Mann-Whitney U tests in SPSS. A significance level of 5% was used.

GSα mutations

Another classification which was used for comparison in this research is the expression level of the target genes in tumors with and without a Gsα mutation. Gsα is a protein subunit which mediates cortisol synthesis and secretion, which occurs after binding of ACTH to the G-protein coupled ACTH receptor in adrenocortical cells. Mutations in the Gsα protein subunit could lead to activation of the subunit.(unpublished results) The expression levels of the target genes in samples with and without a GSα mutation were compared by means of a Mann Whitney-U test. Samples C6, C12, C14, C16, C20, C21, C22, C23, C24, C26, C28, A21, A26, A27 and A29 had a GSα mutation. C7, C9, C13, C17, C19, C27, C30, C31, C32, A22, A23, A24, A25 A28 and A30 did not have this mutation. (c=carcinoma, a=adenoma, n=normal)

2.7 Sequencing

The carcinomas 3,6,8,9,14,15,18,19 and 20 and adenomas 21, 27, 28 and 29 were sequenced to find bRAF mutations. These samples had a fold change of 0,0 or lower of ErbB3. In human thyroid cancer

cells, bRAF mutations were shown to down-regulate ErbB3 expression. These mutations can cause an over-activation of the MAPkinase pathway in human thyroid cancer cells, which leads to a feedback induced suppression of ErbB3 expression.[35] The 9 carcinomas and 4 adenomas in this study were sequenced to know whether bRAF mutations were responsible for the down-regulation of ErbB3 in the canine ATs.

A PCR was used to amplify bRAF isoform 1 from base 1624 to base 1995 (see appendix 6). Gel electrophoresis was used to check whether there is a specific PCR product of the correct product length. (appendix 7). The Big dye terminator cycle sequencing protocol was followed to do the sequencing (appendix 8). The normal sequence of bRAF isoform 1 is depicted in NCBI with the accession number AY545218. The results were analyzed with SeqMan Pro software.

3.RESULTS

Not all the samples could be included in the calculations, because for some samples as Ct-value was not available in the CFX results. Therefore, these samples were not involved in the statistical analysis. Finally, 17 carcinoma-, 9 adenoma- and 9 normal adrenal samples were usable for all genes. There were also a few random samples which had one available Ct-value and one non available Ct-value for a single gene. The available Ct values were used for calculation and statistical analysis.

The samples 2(c) and 24(a) revealed no Ct-values for many reference- and target genes. The samples 10(c), 11(c) and 40(n) had no Ct-values for many target genes. These five samples were excluded for further analysis. Samples 36(n) and 37(n) had high Ct-values of the reference genes in comparison with the other samples. These two control samples were also excluded.

3.1 Reference genes

3.1.1 qPCR and statistical analysis

A reliable set of reference genes was selected for the calculations. Reliable reference genes must have a stable expression in the tissue of interest.

No significant differences with a 5% significance level of the relative expression was observed of HNRPH, RPS5, SRPR, HMBS, SDHA and YWHAZ between the three sample groups. These 6 genes were stable within each group of samples and were selected as reliable reference genes. The CT-values of all reference genes are shown in appendix 9.

3.2 Target genes

3.2.1 qPCR

The mean fold changes of the carcinomas, adenomas and normal samples are shown in figure 1.

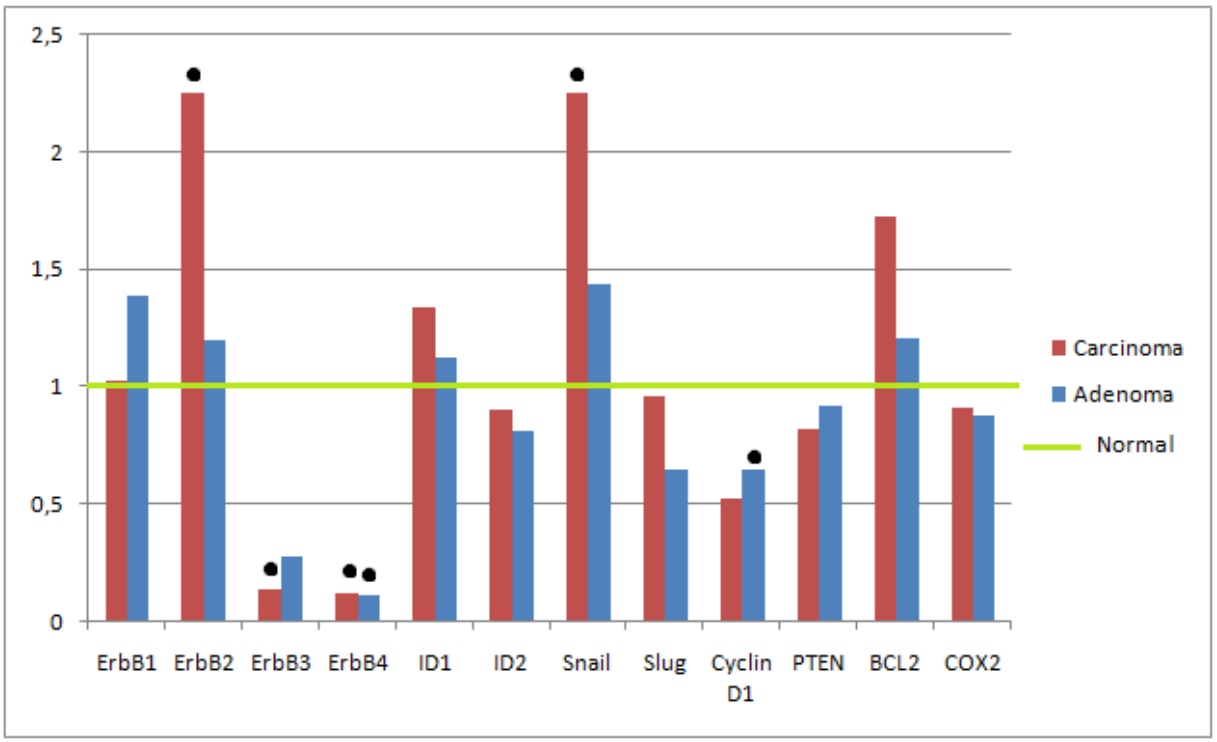

Figure 1: Mean fold changes of the target genes. The columns with a black dot had a significanty different fold change compared to the normal samples, based on a Mann Whitney U test.

3.2.2 Statistical analysis

Table 1,2 and 3 show the results of the statistical analysis.

Comparison	Targetgene	Up(↑)/down(↓)regulation	Expression	Significant
Carcinomas - Normal	ErbB1	↑	1,027	No (p=0,966)
	ErbB2	↑	2,252	Yes (p=0,001)
	ErbB3	↓	0,132	Yes (p=0,007)
	ErbB4	↓	0,118	Yes (p=0,046)
	ID1	↑	1,334	No (p=0,437)
	ID2	↓	0,899	No (p=0,700)
	Snail	↑	2,256	Yes (p=0,027)
	Slug	↓	0,959	No (p=0,898)
	Cyclin D1	↓	0,518	No (p=0,055)

	PTEN	↓	0,816	No (p=0,114)
	BCL2	↑	1,725	No (p=0,076)
	COX2	↓	0,904	No (p=0,832)

Table 1: Statistical analysis: Carcinomas compared with Normal adrenal tissue with a Mann Whitney U test.

Comparison	Targetgene	Up(↑)/down(↓)regulation	Expression	Significant
Adenomas - Normal	ErbB1	↑	1,390	No (p=0,577)
	ErbB2	↑	1,198	No (p=0,685)
	ErbB3	↓	0,272	No (p=0,128)
	ErbB4	↓	0,111	Yes (p=0,039)
	ID1	↑	1,124	No (p=0,753)
	ID2	↓	0,810	No (p=0,341)
	Snail	↑	1,433	No (p=0,258)
	Slug	↓	0,640	No (p=0,255)
	Cyclin D1	↓	0,460	Yes (p=0,011)
	PTEN	↓	0,919	No (p=0,530)
	BCL2	↑	1,204	No (p=0,610)
	COX2	↓	0,876	No (p=0,770)

Table 2: Statistical analysis: Adenomas compared with Normal adrenal tissue with a Mann Whitney U test.

Comparison	Targetgene	Up(↑)/down(↓)regulation	Expression	Significant
Adenomas + Carcinomas / Normal	ErbB1	↑	1,140	No (p=0,838)
	ErbB2	↑	1,810	No (p=0,059)
	ErbB3	↓	0,169	Yes (p=0,021)
	ErbB4	↓	0,115	Yes (p=0,028)

ID1	↑	1,257	No (p=0,479)
ID2	↓	0,867	No (p=0,587)
Snail	↑	1,928	Yes (p=0,047)
Slug	↓	0,834	No (p=0,631)
Cyclin D1	↓	0,497	Yes (p=0,026)
PTEN	↓	0,850	No (p=0,207)
BCL2	↑	1,523	No (p=0,176)
COX2	↓	0,894	No (p=0,782)

Table 3: Statistical analysis: Adenomas + carcinomas compared with Normal adrenal tissue with a Mann Whitney U test.

A significant up-regulation of ErbB2 and Snail was found in the group of carcinomas, when compared to the normal adrenal tissue. The carcinomas had also a up-regulation of ErbB1, ID1 and Bcl-2 compared to the normal adrenal tissue, but this was not significant. The carcinomas had a significant down-regulation of ErbB3 and ErbB4 in comparison with normal adrenals. ID2, Slug, cyclin D1, PTEN and COX2 were non-significantly down regulated compared to the normal tissue. *(Figure 2)*

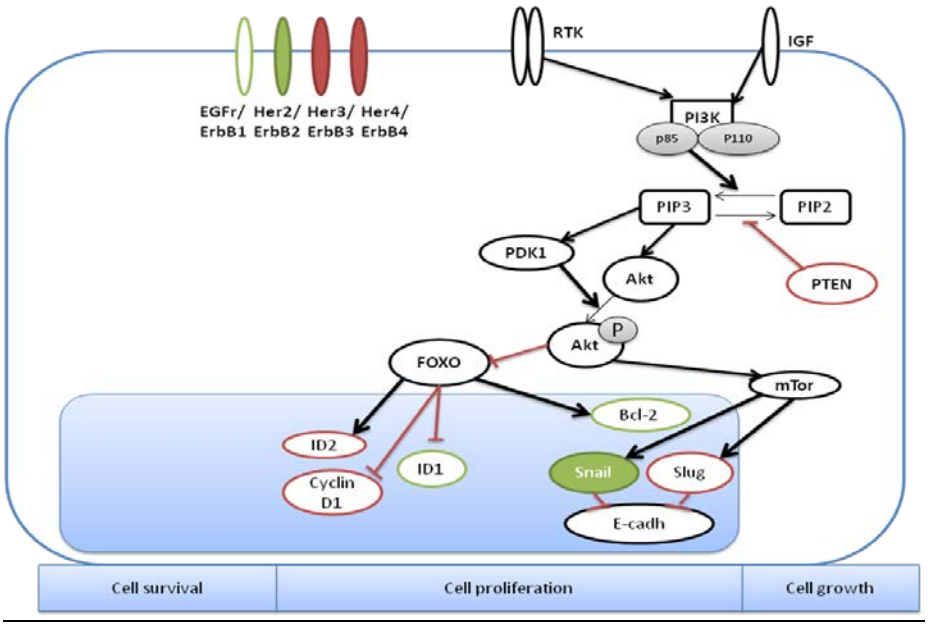

Figure 2: Carcinomas compared to normal adrenals. Black arrows: stimulation/activation, red arrows: inhibition/inactivation, green solid circles: significantly up-regulated, red solid circles: significantly down-regulated, green framed circles: non-significantly up-regulated, red framed circles: non-significantly down-regulated.

There was no significant up-regulation of target genes in the adenoma group compared to the normal adrenal tissue. ErbB1,ErbB2, ID1 Snail and BCL2 were non-significantly up-regulated compared to the normal adrenal tissue. The adenomas showed a significantl down-regulation of ErbB4 and Cyclin D1. ErbB3, ID2, Slug, PTEN and COX2 were no-significantly down-regulated in adenomas. *(Figure 3)*

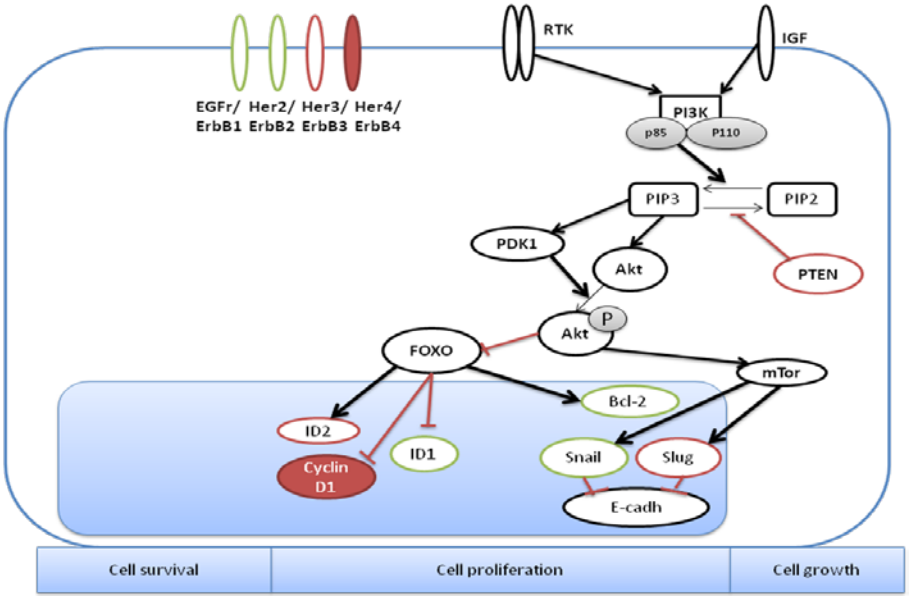

Figure 3: Adenomas compared to normal adrenals. Black arrows: stimulation/activation, red arrows: inhibition/inactivation, green solid circles: significantly up-regulated, red solid circles: significantly down-regulated, green framed circles: non-significantly up-regulated, red framed circles: non-significantly down-regulated.

When all the tumor tissues (the carcinomas and adenomas) were taken together and compared with the normal group, Snail was significantly up-regulated. ErbB1, ErbB2, ID1 and BCL2 were non-significantly up-regulated in the tumors. ErbB3, ErbB4 and Cyclin D1 were significantly down-regulated in the tumors. ID2, Slug, PTEN and COX2 were non-significantly down-regulated compared to the normal adrenal tissue. *(Figure 4)*

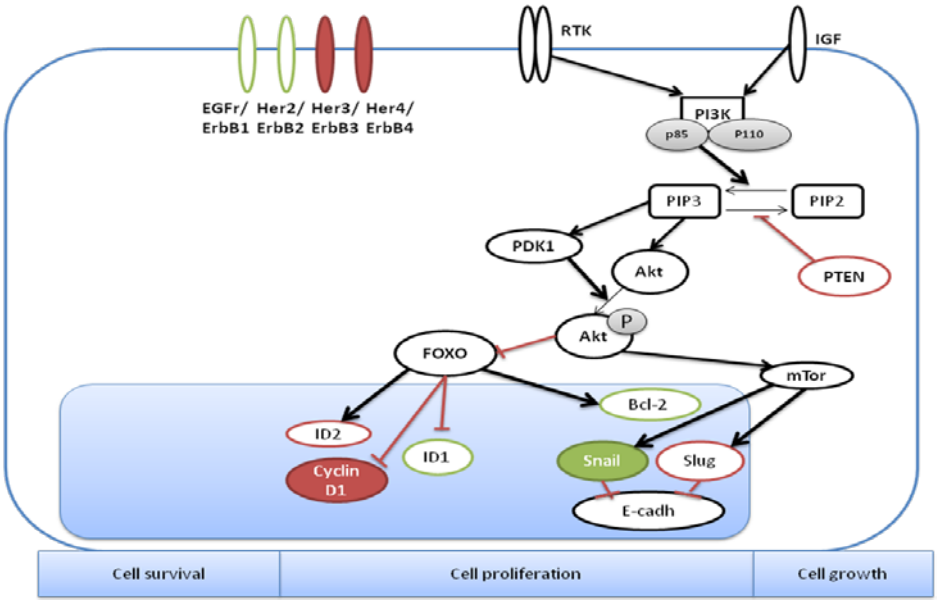

Figure 4: Carcinomas + adenomas compared to normal adrenals. Black arrows: stimulation/activation, red arrows: inhibition/inactivation, green solid circles: significantly up-regulated, red solid circles: significantly down-regulated, green framed circles: non-significantly up-regulated, red framed circles: non-significantly down-regulated.

ErbB2 was significantly up-regulated in the carcinomas compared to the adenomas. (data not shown)

3.2.3 GSα

The mRNA expression of all target genes in samples with a GSα mutation was not significantly different from the mRNA expression in samples with no GSα mutation.

3.3 Sequencing

3.3.1 Gel electrophoresis

The samples which had a fold change of 0,0 or lower in the qPCR of ErbB3 were sequenced for bRAF mutations. The carcinomas C3,C6,C8, C9, C14, C15, C18, C19 and C20 had a fold change of 0,1 or lower. The same applies to the adenomas A21, A27, A28 and A29. There was no PCR product visible in the gel of sample C19. The other samples all had a specific product of approximately 400 base pairs, which corresponds with the expected length of the amplified product. *(figure 5)*

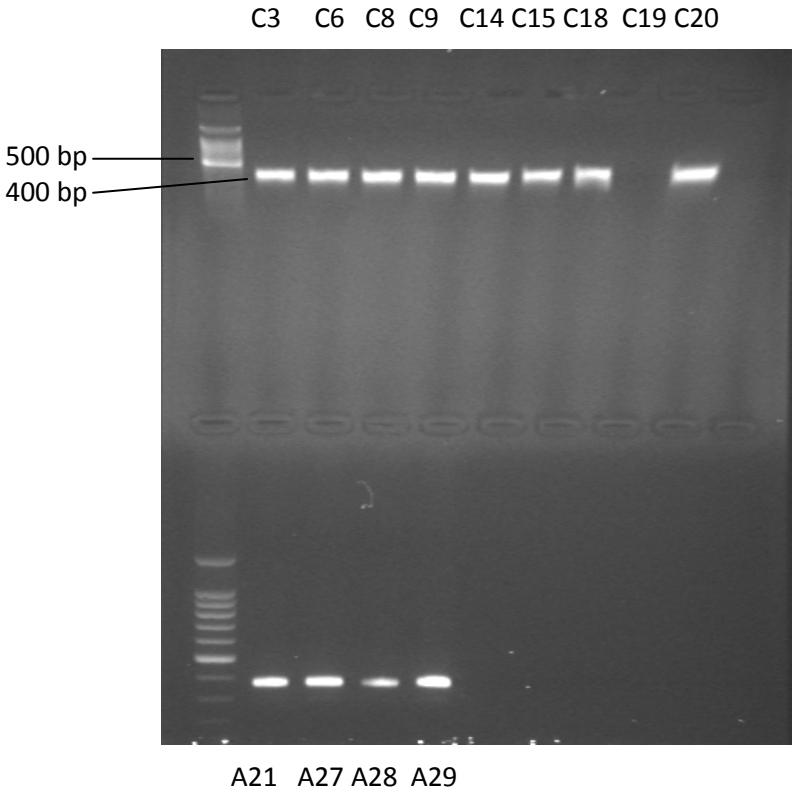

Figure 5: A DNA-ladder of 100 base pairs was used. There was no PCR product visible of sample 19. The other samples had a specific PCR product of approximately 400 base pairs. The upper columns consist of (from the left to the right) DNA ladder, C3, C6, C8, C9, C14, C18, C19 and C20. The lower columns consist of (from the left to the right) DNA ladder, A21, A27, A28 and A29.

3.3.2 Sequence data

There was no sequence data available of sample 18 and 19. A baseline noise was present in the samples with available data (samples 3,6,8,9,14,15,20,21,27,28 and 29). All samples had on several places on single peak positions two peaks instead of just on peak. These 'double peaks' were detected in coding regions in the forward- or reverse strand, but were not present in the complementary peak position. A 'double peak' was detected by the sequencer in sample A28, A29, C3, C8, C14 and C15 on the same position. This 'double peak' on position 1645 consisted of Adenine(A) and cytosine (C). Adenine on this position will lead to the translation of threonine (Thr), while cytosine on this position will lead to the translation of proline (Pro). The sequencer gave no error in the complementary strand on position 1645, but the peak in the complementary strand was not very convincing. Sample A21 had a changed base-pair on position 1902, but it was a silent mutation. Adenine (A) was replaced by guanine (G) in the third base-pair of the codon in the forward and reverse strand. The translation of this codon does not lead to the translation of a different protein in comparison with the reference canine bRAF sequence of exon 15 (appendix 10). The codon still encodes for Threonine(Thr).

4.DISCUSSION

The results of the qPCR give information about the mRNA expression of the investigated genes, but not about the expression of the proteins. Further investigation with an Western blot can give more information about the protein expression of the components of the pathway. A disadvantage of a Western blot is the fact that it is not a quantitative method and less accurate than the qPCR. The qPCR was selected as the best method in this experiment, because it is an accurate quantitative method. Because transcriptional target genes were measured, the mRNA expression gives an indication of the activity of the pathway.

Based on the results of the qPCR, 7 samples were excluded for further analysis. 5 samples revealed no Ct-values for reference- and/or target genes, so something in the cDNA synthesis or PCR went wrong with these samples. 2 samples had high Ct values of the reference genes in comparison with the other samples. Probably something went wrong in the cDNA synthesis, whereby these samples contained less cDNA encoding for the reference genes. The sequencing results revealed 2 samples which revealed no available sequence data. The PCR went wrong for C19, because this sample had no visible PCR product in the gel. C18 had a visible PCR product in the gel, but there was no sequence data available. Probably something went wrong in the sequencing protocol. C18 and C19 have to be sequenced again, because based on the low ErbB3 expression, these samples might contain bRAF mutations.

The results of the qPCR reveal that in canine adrenal tissues the levels of mRNA encoding for ErbB2 and Snail are significantly higher in carcinomas in comparison with the normal adrenal tissue. ErbB2 is associated with a more aggressive clinical behavior and decreased survival rates were observed in Snail-positive tumors in human.[13,27] ErbB2 is overexpressed in many human carcinomas, but no significant overexpression has been found in human ATs.[36,37] The significant overexpression of ErbB2 in canine carcinomas suggests that the ErbB2 receptor could be an interesting target for anticancer therapies targeting ErbB receptors like Herceptin, a monoclonal antibody against ErbB2.[36,38] Further research needs to be done in order to evaluate the potential of these drugs for treatment of canine ATs. The expression of ErbB2 in more cortisol secreting adrenocortical carcinomas should be measured by means of a qPCR. Also the effect of Herceptin on canine cells and ErbB2 receptors in canine cortisol secreting ATs has to be investigated. A Western blot can give more information about the effect of Herceptin on the ErbB2 protein level before and after treatment.[36] The PI3K/Akt/mTOR pathway may be abnormally activated in the investigated canine carcinomas because of the ErbB2 overexpression and ligand independent dimerization. Homodimers of ErbB2 can spontaneously form in ErbB2 overexpressing cells.[16] The overexpression of Snail might be due to activation of the PI3K/Akt/mTOR pathway. In contrast to ErbB2 and Snail, ErbB3 and ErbB4 had a significantly lower expression in the carcinomas, which does not indicate an activation of the pathway due to ErbB3 and ErbB4. A low expression of neuregulin ligands, which only bind to ErbB3 and ErbB4, could explain the low expression of these two receptors since the normal physiological expression and function of the receptors are controlled by the expression of ligands.[15] ErbB3 expression can also be down-regulated due to bRAF mutations. These mutations can cause an over-activation of the MAPkinase pathway in human thyroid cancer cells, which leads to a feedback induced suppression of ErbB3 expression.[35] Activating mutations of bRAF in exon 15 are common in human tumors.[39]. The fact that there were no mutations found in bRAF exon 15 which will lead to the translation of a

different protein compared with the reference sequence, suggests that bRAF mutations in exon 15 are not responsible for the low expression of ErbB3 in the sequenced samples. ErbB1 is also associated with a more aggressive clinical behavior, but does not show a significant overexpression in the carcinoma samples.[13] So ErbB1 does not seems to be an interesting therapeutic target based on the results in this study. Cyclin D1 tended to have a lower expression in the carcinomas in comparison with the normal adrenals, but with a p-value of 0,055 Cyclin D1 did not reached a level of significance.

There was no significant overexpression of target genes in the adenoma tissues in comparison with the normal adrenal tissue. However, the results reveal a significantly lower expression of mRNA encoding for ErbB4 and Cyclin D1. The low expression of ErbB4 may be explained by a low expression of neuregulins. A low expression of CyclinD1 does not match with an activation of the PI3K/Akt/mTOR pathway, because Akt inhibits foxo when the pathway is activated.[19] Since Foxo inhibits Cyclin D1, less inhibition would lead to up-regulation of Cyclin D1 instead of down-regulation .[20] Cyclin D1 is also activated by the Wnt-signaling pathway, so alterations in this pathway can also lead to a low expression.[40] ID1, ID2 and Bcl-2 which are also target of Foxo were not significantly up/down regulated, so Foxo does not seem to be dysregulated.

When all ATs are taken together as one group and this group is compared to the normal adrenal tissue samples, the results reveal a significant overexpression of Snail and a significantly lower expression of ErbB3, ErbB4 and Cyclin D1. The possible explanations of this up- and down-regulations are previously mentioned. ErB2 tended to have a higher expression with a p-value of 0,059, but did not reached a level of significance.

The main difference between the carcinomas and the adenomas is the significant overexpression of ErbB2 in carcinomas. As mentioned earlier, ErbB2 overexpression is associated with a more aggressive clinical behavior in earlier experiments.[13] Significant overexpression of ErbB2 and Snail also seems to be associated with more aggressive clinical behavior in this study since their overexpression occurs in (malignant)carcinomas and not in (benign)adenomas.

The fact that the adenoma group consist of 9 samples and the carcinoma group of 17 samples in the statistical analysis may influence the reliability of the results. The results of the carcinomas are more reliable, because more samples are included. It also means that when the adenomas and carcinomas are taken together as on group and compared with the normal tissue sample in statistical analysis, the carcinomas have a greater influence on the tumor group.

There was no significant difference of the expression of target genes in samples with the GSα mutation in comparison with samples which did not have this mutation. The results of this study suggests that there are no dysregulations of the investigated target genes in the PI3K/Akt/mTOR pathway which declare tumor growth in tumors without GSα mutation.

It should be noted that as mentioned above in the discussion, a higher- or lower expression of the investigated genes does not only have to point to alterations of that specific target gene. The level of ligands or alterations elsewhere in the PI3K/Akt/mTOR pathway but also in the MAPK- or Wnt pathway could lead to an altered expression of the target genes and other genes in the pathway.

5. CONCLUSION

There was a significant overexpression of ErbB2 and Snail in the carcinomas compared to the normal adrenal samples, but there was no obvious activation of the PI3K/Akt/mTOR pathway in this study. Based on the results of this study, it is not clear yet why some gene expressions indicated an activation of the PI3K/Akt/mTOR pathway while other genes expressions did not indicated an activation. Therefore, more research of other pathways which influence the PI3K/Akt/mTOR pathway is needed.

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Appendix 1:

Protocol: Purification of total RNA from animal tissues.

Things to do before starting:

- B-Mercaptoethanol (β-ME) was added to Buffer RLT before use. 10 µl β-ME per 1 ml Buffer RLT was added.

Procedure:

- The procedure for frozen tissues was followed:

  Only a portion of the tissue was used, so the piece was weighed and placed directly into a suitable sized vessel for disruption and homogenization.

- The tissue was disrupted and the lysate was homogenized in Buffer RLT (not more than 30 mg tissue was used) according to the next step.

- The tissue was disrupted and homogenized using a rotor-stator homogenizer:

  The weighed frozen tissue was placed in a suitably sized vessel. 700 µl RLT was added. The tissue was immediately disrupted and homogenized using a conventional rotor-stator homogenizer until it was uniformly homogeneous (usually 20-40 s).

- The lysate was centrifuged for 3 min at full speed. The supernatant was carefully removed by pipetting, and transferred to a new microcentrifuge tube. Only this supernatant (lysate) was used in subsequent steps.

- 1 volume of 70% ethanol was added to the cleared lysate, and mixed immediately by pipetting.

- Up to 600 µl of the mix, including any precipitate that may have formed, was transferred to an RNeasy spin column placed in a 2 ml collection tube. The spin column was centrifuged for 15 s at ≥8000 x g (≥10.000 rpm). The flow-trough was discarded.

Now, the optional on-column DNase digestion procedure with the RNase-free DNase set was followed:

- 350 µl Buffer RW1 was added to the RNeasy spin column. The spin column was centrifuged for 15 s at 8000 x g (10.000 rpm) to wash the spin column membrane. The flow-through was discarded.

- 10 µl DNase I stock solution was added to 70 µl Buffer RDD. It was mixed by gently inverting the tube, and centrifuged briefly to collect residual liquid from the sides of the tube.

- 80 µl DNase I incubation mix was added directly to the RNeasy spin column mebrane, and placed on the benchtop (20-30^{o}C) for 15 min.

- 350 µl Buffer RW1 was added to the RNeasy spin column. The spin column was centrifuged for 15 s at 8000 x g (10.000 rpm). The flow-through was discarded.

Now, the purification of total RNA from animal tissues protocol was resumed.

- 500 µl Buffer RPE was added to the RNeasy spin column. The spin column was centrifuged for 15 s at ≥ 8000 x g (≥10.000rpm) to wash the spin column membrane. The flow-through was discarded.
- 500 µl Buffer RPE was added to the RNeasy spin column again. The spin column was centrifuged for 2 min at ≥ 8000 x g (≥10.000rpm) to wash the spin column membrane.
- The RNeasy spin column was placed in a new 2 ml collection tube, and the old collection tube with the flow-through was discarded. The spin column was centrifuged at full speed for 1 min.
- The RNeasy spin column was placed in a net 1,5 ml collection tube. 30 µl RNase-free water was added directly to the spin column membrane. The spin column was centrifuged for 1 min at ≥ 8000 x g (≥10.000rpm) to elute the RNA.

Appendix 2:

Protocol: cDNA synthesis

The cDNA was made using the iScript^{TM}cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Appendix 3 gives an overview of the all the samples that were used. For cDNA synthesis, a starting quantity of 1000ng RNA was used. This means that the amount of isolated RNA solution which has to be added in the tube differs in each sample and it is necessary to measure the amount of RNA of each sample with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). When the RNA concentration in a sample was very high, it was necessary to dilute the RNA sample 10x. MilliQ was added to 1000ng RNA to add up to a volume of 15ul in each tube. Thereafter, 4 µl iScript Reaction Mix and 1µl iScript reverse transcriptase were added to each sample.

The tubes with reverse transcriptase were the reverse transcriptase positive (RT +) tubes. Reverse transcriptase negative (RT-) tubes of each sample were also made to monitor for potential DNA contamination. These tubes did not contain reverse transcriptase. The reaction setup in the table below was used to make the RT-, but 1µl milliQ was added to the tube instead of 1µl reverse transcriptase.

Finally, the RT+ and RT- tubes of the 40 samples were placed in the PCR C1000^{TM} Thermal Cycler (Bio-Rad, CA, USA)

The reaction set up was as follows:

Component	Volume per reaction
5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	X µl
RNA template (100fg to 1µ Total RNA)	X µl
Total volume	20µl

Appendix 3

Sample nr Carcinomas	RNA concentration (ng/µl)	µl RNA (10x diluted)	µl MilliQ
1 (c)	600,10	8,33	6,67
2 (c)	657,63	7,60	7,40
3 (c)	273,40	1,83	13,17
4 (c)	717,08	6,97	8,03
5 (c)	174,00	2,87	12,13
6 (c)	295,10	1,69	13,31
7 (c)	1440,47	3,47	11,53
8 (c)	695,02	7,19	7,81
9 (c)	266,60	1,88	13,12
10 (c)	751,50	6,65	8,35
11 (c)	581,00	0,86	14,14
12 (c)	402.80	1,24	13,76
13 (c)	110,70	4,52	10,48
14 (c)	804,40	6,22	8,78
15 (c)	564,70	0,89	14,11

16 (c)	348,30	1,44	13,56
17 (c)	361,10	1,38	13,62
18 (c)	224,44	2,23	12,77
19 (c)	539,00	9,28	5,72
20 (c)	851,90	5,87	9,13

Sample nr **Adenomas**	**RNA concentration (ng/µl)**	**µl RNA** **(10x diluted)**	**µl MilliQ**
21 (a)	292,90	1,71	13,29
22 (a)	81,80	6,11	8,89
23 (a)	510,50	9,79	5,21
24 (a)	1172,40	4,26	10,74
25 (a)	785,85	6,36	8,64
26 (a)	1225,34	4,08	10,92
27 (a)	1291,62	3,87	11,13
28 (a)	682,67	7,32	7,68
29 (a)	784,60	6,37	8,63
30 (a)	440,30	1,14	13,86

Sample nr **Healthy adrenocortical tissue**	**RNA concentration (ng/µl)**	**µl RNA** **(10x diluted)**	**µl MilliQ**
31 (n)	1365,00	3,66	11,34
32 (n)	1345,05	2,72	11,28
33 (n)	525,70	9,51	5,49
34 (n)	612,20	8,17	6,83

35 (n)	1718,62	2,91	12,09
36 (n)	401,00	1,25	13,75
37 (n)	754,00	6,63	8,37
38 (n)	662,90	0,75	14,25
39 (n)	262,84	1,90	13,10
40 (n)	278,20	1,80	13,20

Appendix 4

Targetgene	**Annealing temperature (C°)**	**2 Step**	**Primer sequence (5'-3')**
EGF1	53	no	Fw: Rv:
EGF2	64	yes	Fw: Rv:
EGF3	64	yes	Fw: Rv:
EGF4	70	yes	Fw: Rv:
ID-1	59,5	yes	Fw: CTCAACGGCGAGATCAG Rv: GAGCACGGGTTCTTCTC
ID-2	60,5	yes	Fw: GCTGAATAAATGGTGTTCGTG Rv: GTTGTTCTCCTTGTGAAATGG
Snail	61,5	yes	Fw: CAAGATGCACATCCGAAGC Rv: GCAGTGGGAGCAGGAAAAC
Slug	60	yes	Fw: CTTCACTCCGACTCCAAACG Rv: TGGATTTTGTGCTCTTGCAG
Cyclin D1	60	yes	Fw: ACTACCTGGACCGCT Rv: CGGATGGAGTTGTCA
PTEN	62	yes	Fw: AGATGTTAGTGACAATGAACCT Rv: GTGATTTGTGTGTGVTGATC
BCL-2	62	yes	Fw: GGAGAGCGTCAACCGGGAGATGT Rv: GGTGTGCAGATGCCGGTTCAGGT
COX2	60	yes	Fw: TTCCAGACGAGCAGGCTAAT Rv: GCAGCTCTGGGTCAAACTTC

Reference gene	Annealing temperature (C°)	2 Step	Primers
GAPDH	58	yes	Fw: TGTCCCCACCCCCAATGTATC Rv: CTCCGATGCCTGCTTCACTACCTT
HPRT	56+58	yes	Fw: AGCTTGCTGGTGAAAAGGAC Rv: TTATAGTCAAGGGCATATCC
RPS19	61+63	yes	Fw: CCTTCCTCAAAAAGTCTGGG Rv: GTTCTCATCGTAGGGAGCAAG
HNRPH	61,2	yes	Fw: CTCACTATGATCCACCACG Rv: TAGCCTCCATAACCTCCAC
RPL8	55	no	Fw: CCATGAATCCTGTGGAGC Rv: GTAGAGGGTTTGCCGATG
GUSB	62	yes	Fw: AGACGCTTCCAAGTACCCC Rv: AGGTGTGGTGTAGAGGAGCAC
RPS5	62,5	yes	Fw: TCACTGGTGAGAACCCCCT Rv: CCTGATTCACACGGCGTAG
SRPR	61,2	yes	Fw: GCTTCAGGATCTGGACTGC Rv: GTTCCCTTGGTAGCACTGG
B2MG	61,2+63	yes	Fw: TCCTCATCCTCCTCGCT Rv: TTCTCTGCTGGGTGTCG
HMBS	61	yes	Fw: TCACCATCGGAGCCATCT Rv: GTTCCCACCACGCTCTTCT
RPL13	61	yes	Fw: GCCGGAAGGTTGTAGTCGT Rv: GGAGGAAGGCCAGGTAATTC
SDHA	61	yes	Fw: GCCTTGGATCTCTTGATGGA Rv: TTCTTGGCTCTTATGCGATG
YWHAZ	58	yes	Fw: CGAAGTTGCTGCTGGTGA Rv: TTGCATTTCCTTTTTGCTGA
TBP	57	no	Fw: CTATTTCTTGGTGTGCATGAGG Rv: CCTCGGCATTCAGTCTTTTC

Appendix 5

Protocol: qPCR iQSYBRgreensuperMix

- Primer solutions

  New primers were dissolved in milliQ (mQ) to obtain a primary stock solution with a concentration of 100 pmol/µl. The working stocks were 10x dilutions of the primary stocks obtaining a 10pmol/µl concentration.

- Sample dilution

First, the sample dilution was made by diluting the cDNA 10x. In the previous cDNA synthesis step, 20µl cDNA was made of each sample. To make the 10x dilution, 180µl MilliQ was added to each 20µl cDNA. A pool of 10 randomly selected RT- adrenal samples was made to make 4 RT- samples.

- Standard dilution

  Approximately 10 µl of S1 is needed per gene of interest, so the volume of the pool cDNA depends on the numbers of genes. In this study there were 25 genes of interest, but the standard dilution is based on 30 genes in this experiment. 3 µl is extracted from each sample tube to make the standard pool. The highest standard (S1) consists of all of the 97 samples (insulinomas + adrenal tissue). The standard dilutions ranged from Standard 1 (S1) till Standard 8 (S8). The serial dilution for the standard line was a 4 fold dilution.

- Sample dilution 2

  Finally, a 50x dilution of cDNA is needed. The cDNA was already 10x diluted, so a 5x dilution was made. 8 µl 50x diluted sample is needed per gene. A total of 300 µl was made in this experiment to make sure there was enough diluted cDNA. 240 µl mQ was added to each 60 µl 10x diluted sample.

- Performing duplo 10 µl reactions (384 well format)

  The master mix was prepared as in the table below (Standards are included in calculation). This master mix was made for each 384 wells plate.

Samples	117 x duplo
MQ	52 µl
2x iQ SYBR green SuperMix	1287 µl
10 µM Forward Primer	103 µl
10 µM Reverse Primer	103 µl

6,0 µl master mix was pipetted to each well of a BioRad I-Cycler 384 wells plate. Then, 4,0 µl diluted (50x) cDNA was added to each well.

Overview wells plates:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	S1	INS	INS	INS	INS	INS	INS	INS	INS	C 1	C 9	C 17	A 25	N 33	RT-1
B	S1	INS	INS	INS	INS	INS	INS	INS	INS	C 1	C 9	C 17	A 25	N 33	RT-1
C	S2	INS	INS	INS	INS	INS	INS	INS	RT-	C 2	C 10	C 18	A 26	N 34	RT-2
D	S2	INS	INS	INS	INS	INS	INS	INS	RT-	C 2	C 10	C 18	A 26	N 34	RT-2
E	S3	INS	INS	INS	INS	INS	INS	INS	RT-	C 3	C 11	C 19	A 27	N 35	RT-3
F	S3	INS	INS	INS	INS	INS	INS	INS	RT-	C 3	C 11	C 19	A 27	N 35	RT-3
G	S4	INS	INS	INS	INS	INS	INS	INS	RT-	C 4	C 12	C 20	A 28	N 36	RT-4
H	S4	INS	INS	INS	INS	INS	INS	INS	RT-	C 4	C 12	C 20	A 28	N 36	RT-4
I	S5	INS	INS	INS	INS	INS	INS	INS	RT-	C 5	C 13	A 21	A 29	N 37	NTC
J	S5	INS	INS	INS	INS	INS	INS	INS	RT-	C 5	C 13	A 21	A 29	N 37	NTC
K	S6	INS	INS	INS	INS	INS	INS	INS	RT-	C 6	C 14	A 22	N 30	N 38	
L	S6	INS	INS	INS	INS	INS	INS	INS	RT-	C 6	C 14	A 22	N 30	N 38	
M	S7	INS	INS	INS	INS	INS	INS	INS	RT-	C 7	C 15	A 23	N 31	N 39	
N	S7	INS	INS	INS	INS	INS	INS	INS	RT-	C 7	C 15	A 23	N 31	N 39	
O	S8	INS	INS	INS	INS	INS	INS	INS	RT-	C 8	C 16	A 24	N 32	N 40	
P	S8	INS	INS	INS	INS	INS	INS	INS	RT-	C 8	C 16	A 24	N 32	N 40	

Figure x: The standard dilution was pipette into the first column. Column 2 till 9 contained insulinoma samples of another experiment. A10 - H12 contained the carcinomas, I12 - J13 the adenomas, K13 – P14 the normal adrenal tissues, A15 – H15 the reverse transcriptase negative samples and I15 – J15 the non template control. All the samples were in duplo.

- After the plate was sealed, it was centrifuged for 5 seconds. Finally, the plate was placed in qPCR device. (C1000 Touch Thermal Cycler, CFX 384™ Real-Time System) (Bio-Rad, CA, USA). The annealing temperatures of the genes are shown in the table of appendix x. The results of the qPCR were analyzed with Bio-Rad CFX Manager™ 3.0.

Appendix 6

Protocol: PCR

The following protocol was used for the PCR amplification of bRAF. The reaction set up was as follows:

Mix	**Per sample (µl)**
MQ	12,4
HF buffer	4
Forward primer (10x diluted) Fw:	1
Reverse primer (10x diluted) Rv:	1
dNTP	0,4
Phusion Taq	0,2

Total	**19**

- 1µl cDNA was added to each 19µl reaction mix.
- the samples with cDNA and reaction mix were placed in the C1000 thermal cycler (Bio-Rad, CA, USA). The C1000 thermal cycler followed the program depicted below:

1	2	3	4	5	6	7
98,0 °C 0:30	98,0 °C 0:10	50,0 °C 0:20	72,0 °C 0:15	Go To 2 39x	72,0 °C 5:00	20,0 °C Forever

Appendix 7

Protocol: gel electrophoresis

- To make the gel, 0,6 gram agarose was added to 60ml TBE buffer.
- The buffer with agarose was heated in the microwave until it boiled. The solution was swirled until it was a clear solution. The clear solution was cooled until it was handwarm.
- 5 µl ethidiumbromide was added. The solution was poured into the gel tray and the height combs were placed in the gel tray. The agarose was solidified after about 15-30 minutes.
- The height combs were removed when the agarose gel was solidified. The gel tray was placed in the agarose gel electrophoresis system.
- 5µl 100bp ladder DNA ladder was mixed with 2µl loading dye and added in the first lane.
- 10µl sample mixed with 2µl loading dye was pipetted in the following lanes. The gel was run on 100V.
- After 15-30 minutes the DNA bands were separated by size and the DNA was visible under UV-light in the GelDoc.

Appendix 8

Protocol: Big dye terminator cycle sequencing

- First, the sequencing reaction had to be prepared. The reaction set up was as follows:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>For each reaction, the following reagents were added to a separate tube:</td></tr>
<tr><td></td><td>
<table>
<tr><td>Terminator Ready Reaction Mix</td><td>1 µ</td></tr>
<tr><td>Template:

PCR product (10x diluted)</td><td>3 µl</td></tr>
<tr><td>Primer, 3.2 pmol</td><td>1 µl</td></tr>
<tr><td>5x Sequence buffer</td><td>2 µl</td></tr>
<tr><td>MilliQ</td><td>3 µl</td></tr>
<tr><td>Total volume</td><td>10 µl</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>The tubes were mixed well.</td></tr>
</table>

- The 13 tubes containing the PCR product of carcinomas 3,6,8,9,14,15,18,19 and 20 and adenomas 21, 27, 28 and 29 were placed in the DYAD Disciple thermal cycler (Bio-Rad, CA, USA). The DYAD Disciple thermal cycler followed the program below:

Step	Action
1	The following was repeated for 25 cycles: - Rapid Thermal ramp to 96 ^{o}C - 96 ^{o}C for 30 sec. - Rapid thermal ramp to 50 ^{o}C - 55 ^{o}C for 15 sec. - Rapid thermal ramp to 60 ^{o}C - 60 ^{o}C for 30 sec.

2	There was a rapid thermal ramp to 4 ^{o}C and samples were held at this temperature until purification.

- The contents of the tubes were centrifuged for about 5 seconds.
- A MultiScreen MAHV 96- well filtration plate was used for sequencing. The 96 well plate was already prepared and contained Sephadex. The plate was ready to use.
- 20 µl milliQ water was added to the sequencing reactions (10 µl). Everything was pipetted to the center of the columns.
- The MAHV plate was placed on top of a sequencing plate (an MicroAMP® Optical 96- Well Reaction Plate) and centrifuged at 1900 rpm for 5 min.
- Then, the plate was placed in the 3130XL sequencer (Azco Biotech Inc, CA, USA).
- The results of the sequencing were analyzed with DNAstarLaserGene 8 software.

Appendix 9

Reference gene	**Carcinomas** **Ct -values**	**Adenomas** **Ct -values**	**Normal** **Ct -values**
GAPDH	19,7	19,8	20,6
HPRT	23,9	23,7	24,1
RPS19	19,4	19,7	19,8
HNRPH	23,8	24,1	24,2
RPL8	19,3	19,5	19,4
GUSB	25,9	25,9	26,8
RPS5	20,0	20,4	20,6
SRPR	25,3	25,5	26,0
HMBS	25,1	25,0	25,4
RPL13	19,8	20,1	20,0

SDHA	23,6	23,4	24,0
YWHAZ	22,3	22,2	22,8
TBP	27,2	27,3	28,0
B2MG	20,0	20,0	19,8

Table 1: Ct-values of the reference genes. HNRPH, RPS5, SRPR, HMBS, SDHA and YWHAZ were selected as a reliable set of reference genes based on the results in geNorm and REST at the 5% significance level.